– Review paper

Plant Tissue Cult. **12**(2) : 181-194, 2002 (December)

PTC

The Unique Auxin Influx Modulator Chromosaponin I : A Physiological Overview

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Chromosaponin I (CSI) is a g-pyronyl-triterpenoid saponin (Fig. 1), isolated from pea (Tsurumi et al. 1991, 1992) and other leguminous plants (Kudou et al. 1992, 1993; Massiot et al. 1992). Although CSI is present at a relatively high concentration (2 - 3 mM) in the meristematic tissue of pea seedlings (Tsurumi et al. 1992), no distinct physiological role for this endogenous saponin in growth and development of plants has been established so far. Some studies showed that CSI stimulates the growth of roots in a variety of plants (Tsurumi and Wada 1995) and increases the mechanical extensibility of cell wall (Tsurumi et al. 1996). Involvement of ethylene in CSI action has been suggested (Tsurumi and Ishizawa 1997; Rahman et al. 2000). Other saponins have also been shown to influence some biological processes as reviewed by Geuns (1978). Our studies have revealed that CSI modulates auxin influx in root cells of the model plant *Arabidopsis thaliana* by interacting with the auxin influx carrier AUX1 (Rahman et al. 2001a). In the present review we mainly focus on the physiological activity of CSI on auxin influx and ethylene responses in the roots of *Arabidopsis* seedlings. We also briefly introduce the interesting effect of CSI on the sugar taste receptor cell in the blowfly, *Phormia regina,* another model system for studying signal transduction cascade in invertebrates.

CSI stimulates cell elongation and cell division in Arabidopsis roots grown horizontally : The relatively small genome size, the ease of isolation and characterization of mutants make *Arabidopsis thaliana* a widely accepted model plant for studying the mechanism of growth and development. The extreme sensitivity of *Arabidopsis* roots towards CSI along with the availability of several well-characterized hormone related mutants (Ecker 1995) make it an attractive study material to analyze the mechanism of CSI action in growth and development. This is despite the fact that it does not seem to have this type of saponin (unpublished data).

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When *Arabidopsis* seedlings were grown on wet filter paper in the light, the growth rates of roots in Columbia (Col) and Landsberg erecta (Ler) wild-types were 0.92 and 0.26 mm/d, respectively. However, in the presence of 300 μM CSI the growth rates were accelerated to 3.46 (Col) and 2.20 (Ler) mm/d; stimulation in growth rate reached 3.7-fold in Col and to 8.5-fold in Ler. The length of mature epidermal cells was increased by 1.8-fold (Col) and 2.81-fold (Ler) compared with the control and the number of epidermal cells also increased by a factor of 1.65 (Col) and 2.12 (Ler). Such great effects of saponins on the growth of plants have never been reported (Rahman et al. 2000).

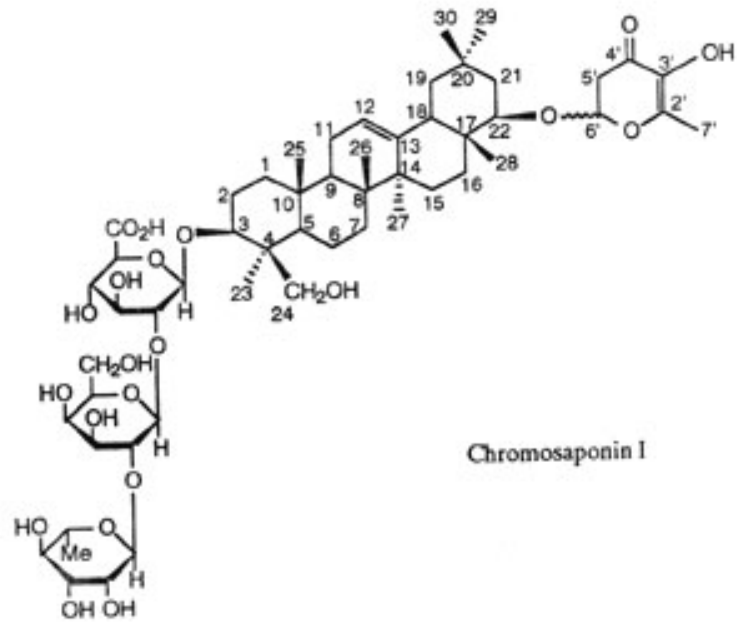

Fig. 1. Chemical structure of CSI (M.W.1068), 3-*O*-[*α*-L-rhamnopyranosyl-(1⊥2)-*β*-D-galactopyranosyl (1 ⊥ 2) - *β*- D-glucuronopyranosyl (1 ⊥)] - 22 - *O* - [3' -hydroxy-2'-methyl -5',6'-dihydro -4'-pyrone(6 ⊥)]-3*β*, 22*β*, 24-trihydroxyolean-12-ene.

One of the interesting effects of CSI is to make the root cells slender. CSI treatment reduced the diameter of root cells as well as increased cell length, suggesting that CSI promotes the cell growth longitudinally. This effect on cell shape can be obtained by blocking the ethylene production or ethylene perception in root cells. Indeed the ethylene biosynthesis inhibitor, 2-aminoethoxyvinyl-glycine (AVG), also increased cell length and decreased cell diameter as CSI did (Rahman et al. 2000). This raised the possibility that CSI may inhibit ethylene production or action. Analysis of the levels of

ethylene revealed that CSI did not inhibit its production (Rahman et al. 2000). Moreover, effects of CSI on root growth were not detected in the ethylene-insensitive mutant *ein2-1*. However, CSI stimulated the growth of roots in *ctr1-1*, where ethylene signaling is constitutively activated (Kieber et al. 1993).

Ethylene signaling is disrupted in *ein2-1* mutant (Guzmán and Ecker 1990) because of a mutation in the bifunctional transducer protein EIN2 (Alonso et al. 1999). The Raf kinase homolog CTR1 is predicted to negatively regulate the ethylene response pathway through a MAP kinase cascade (McGrath and Ecker 1998). The ethylene receptor ETR1 activates CTR1 in the absence of ethylene and is negatively regulated by ethylene (Hua and Meyerowitz 1998). Collectively, these results suggest that inhibition of ethylene signaling may be the cause of CSI-induced cell elongation and that CSI inhibits ethylene signaling at or downstream of the site for CTR1 (Rahman et al. 2000). The mechanism in other responses to CSI action such as increase in cell number, is not yet clear but possible involvement of gibberellin signaling has been shown (Rahman et al. 2000).

CSI specifically interacts with AUX1 protein in regulating the gravitropic and ethylene responses in Arabidopsis roots : Horizontally grown wild-type *Arabidopsis* roots exhibited an uneven growth pattern in the absence of CSI, while CSI-treated wild-type roots grew straight with an enhanced elongation (Tsurumi et al. 2000). This CSI-induced straight growth of wild type roots is similar to the root phenotype of some agravitropic mutants including *aux1-7* (Pickett et al. 1990). Surprisingly, CSI-treatment of the horizontally grown *aux1-7* roots resulted in the same uneven growth pattern as if they restored the gravity response to the levels of wild-type roots. The completely opposite effects of CSI on the growth patterns of wild-type versus *aux1-7* roots suggest that CSI may affect the root gravitropic response. Hence, the effect of CSI on root gravitropism was investigated in detail (Rahman et al. 2001a). The *AUX1* gene encodes an amino acid permease-like protein, which has been shown to be an auxin influx carrier in roots, and *aux1-7* roots show a reduced auxin uptake (Bennett et al. 1996; Yamamoto and Yamamoto 1998; Marchant et al. 1999; Rahman et al. 2001a).

Application of 60 μM CSI disrupted the vertically oriented elongation of wild-type roots and some roots grew horizontally. In contrast, CSI-treatment of *aux1-7* seedlings resulted in elongation of those agravitropic roots towards gravity as if they were wild-type (Fig. 2A). Fig. 2B represents a typical picture of CSI-induced restoration of gravitropic response in *aux1-7* roots. It is

interesting to note that CSI-treated *aux1-7* roots started to bend towards gravity within 2 hr of gravity stimulation, which is similar to that of wild-type root-tip bending, while the untreated *aux1-7* root did not bend towards gravity even after 24 hr (Fig. 2B; Rahman et al. 2001a). This interesting effect of CSI was also observed in the dark, confirming that CSI modulates gravitropic but not phototropic response in roots (Rahman et al. 2001a).

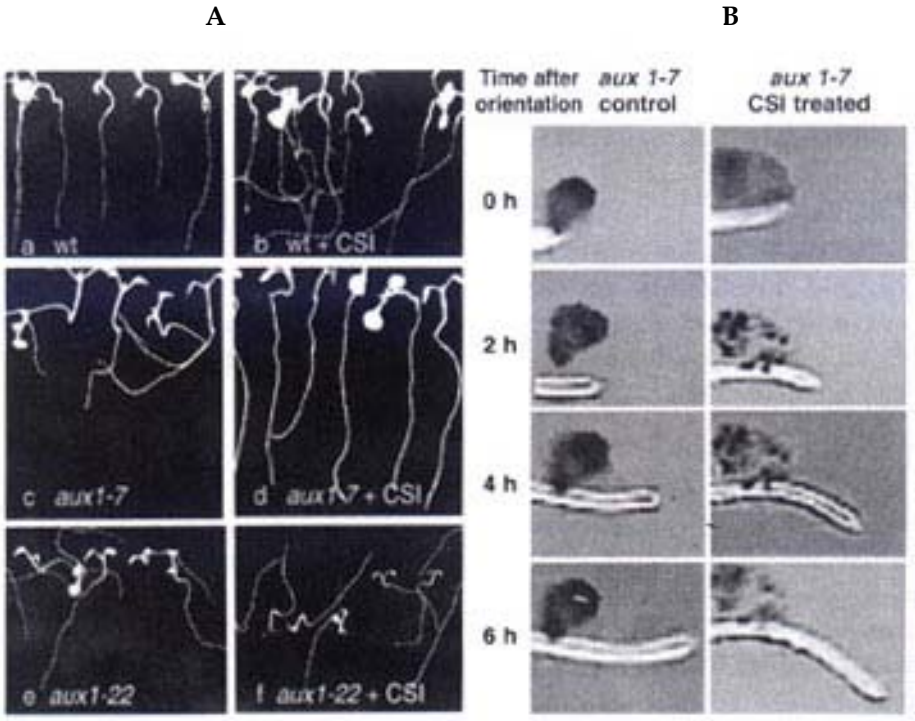

Fig. 2. Effect of CSI on the root growth orientation. **A**, CSI disrupts the vertically oriented growth of wild-type roots (a and b) but orients the growth of *aux1-7* roots towards the gravity (c and d), while in *aux1-22* roots CSI fails to induce any change (e and f). *Arabidopsis* seedlings were grown on vertical agar plates in the presence (b, d and f) or absence (a, c and e) of 60 μM CSI under continuous irradiation for four days. Bar represents 1 mm. This figure is copyrighted by the American Society of Plant Biologists and reprinted with permission. **B**, typical CSI-induced restoration of gravitropic response in *aux1-7* roots (right) compared with control (left). Black marks were given on the surface of plastic Petri dish to indicate the initial position of root tip just after gravistimulus (0 h).

Because the *aux1-7* mutant shows reduced sensitivity to auxin and ethylene, the effects of CSI on another auxin-resistant mutant, *axr1-3* (Lincoln et al. 1990) and ethylene-insensitive mutant *ein2-1* were also investigated. In the wild-type, *axr1-3* and *ein2-1* roots, CSI slowed down the rates of gravitropic bending and inhibited [^{3}H] indole-3-acetic acid (IAA) uptake (Fig. 3A).

In contrast, in *aux1-7* roots, CSI partially restored the uptake of IAA and induced gravitropic bending (Figs. 2 and 3A). This close correlation between auxin uptake and gravitropic bending suggests that CSI regulates gravitropic response by inhibiting or stimulating the uptake of endogenous auxin in root cells. Furthermore, in contrast to IAA, CSI did not influence the uptake of 1-naphthaleneacetic acid (NAA; Fig. 3B). Since NAA is believed to enter the

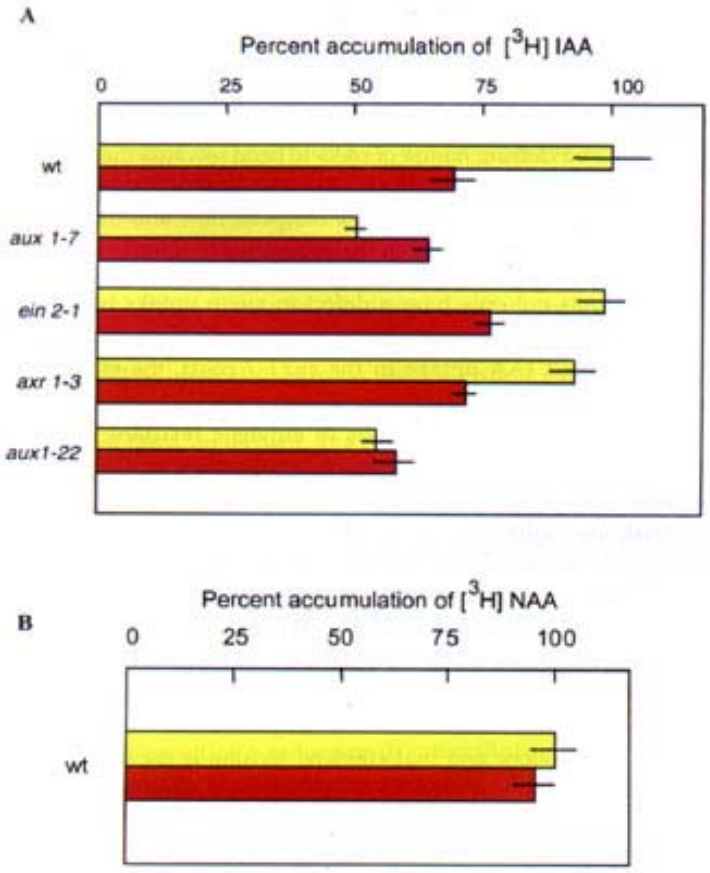

Fig. 3. Effect of CSI on the uptake of [^{3}H]IAA (**A**) and [^{3}H]NAA (**B**) in the root tips of wild-type, *aux1-7*, *ein2-1*, a*xr1-3* and *aux1*-22. Arabidopsis seedlings were grown on vertical agar plate with (red bar) or without (yellow bar) 60 μM CSI for 4 days in the light. Ten root tips of 3 mm in length were incubated with 30 nM [^{3}H]IAA or [^{3}H]NAA for 1 h. After the incubation, root tips were washed and the radioactivity was counted. Data are the averages of 12 experiments. 100% means the accumulation of labeled IAA or NAA in wild-type roots.

cells through diffusion (Delbarre et al. 1996; Yamamoto and Yamamoto 1998; Marchant et al. 1999), the selective effect of CSI towards IAA is a good piece of evidence for the interaction of CSI with the auxin influx carrier protein (Rahman et al. 2001a).

Recently, Rashotte et al. (2000) showed that the basipetal transport of auxin from the root tip to the elongation zone is required for gravitropic response of *Arabidopsis* roots. The most reasonable model for the agravitropic nature of *aux1-7* roots may be as follows; the mutation in AUX1 protein reduces auxin uptake and thereby reduces endogenous auxin level in root cells. As a result, the amount of auxin transported from the root tip towards the elongation zone may be reduced causing failure of roots to bend towards the gravity. Effects of CSI on gravitropic response fit this model. The inhibition of auxin uptake in wild-type root results in a reduction in its basipetal flow and the restoration in auxin uptake of *aux1-7* roots results in the restoration its flow.

The *aux1* roots not only have a defect in auxin uptake but are also less sensitive to inhibition of root growth by ethylene (Pickett et al. 1990). Since CSI partially restored IAA uptake in the *aux1-7* roots, the effects of CSI on ethylene sensitivity of this mutant were also examined. As expected, CSI also restored ethylene response in the roots of ethylene resistant mutant *aux1-7* (Rahman et al. 2001a). On the contrary, application of CSI to wild-type seedlings made the roots more resistant to exogenous ethylene, specially at a low concentration of ethylene. The simplest explanation for the restoration of ethylene response in *aux1-7* roots is that a certain level of auxin in root cells is required for ethylene response which the CSI treatment provided in the *aux1-7* root cells by stimulating auxin uptake (Fig. 4D). In contrast, application of CSI to wild-type seedlings reduced auxin uptake in roots and made the roots more resistant to ethylene (Fig. 4B). This idea implies that the reduction of intracellular level of auxin may be, at least in part, the cause of the resistance of *aux1-7* roots to ethylene and that ethylene sensing is regulated by the level of endogenous auxin in root cells. This hypothesis is further examined in the next section.

Lastly and most convincingly, in the null allele of *aux1*, *aux1-22* (Marchant and Bennett 1998), whose phenotypes include agravitropic nature of roots, CSI had no effect on the reduced uptake of IAA and resistance to ethylene. All of these results are consistent with the idea that the AUX1 protein is the carrier for auxin uptake and CSI specifically interacts with this protein (Rahman et al. 2001a).

Auxin is a positive regulator for ethylene-mediated response in the growth of Arabidopsis roots : The requirement for auxin in the ethylene-mediated growth response in roots of *Arabidopsis* seedlings was further examined (Rahman et al. 2001b) using two ethylene-resistant mutants, *aux1-7* and *eir1-1*. The auxin efflux mutant *eir1* was originally isolated on the basis of ethylene resistance in its root growth (Roman et al. 1995). Later, it was found that the *EIR1/AGR1/AtPIN2/WAV6* gene encodes an auxin efflux carrier in *Arabidopsis* roots (Chen et al. 1998; Luschnig et al. 1998; Müller et al. 1998; Utsuno et al. 1998).

Application of 10 nM IAA to *aux1-7* roots did not change the resistance towards ethylene, while in the presence of 10 nM NAA the growth inhibition in *aux1-7* roots by ethylene was restored almost to the level in wild-type roots. The selective influence of NAA versus IAA on the restoration of ethylene response in *aux1-7* roots suggests that an increase in the intracellular level of auxin is required for ethylene response in this mutant root. On the other hand, application of 10 nM IAA or NAA restored the ethylene-mediated response in *eir1-1* roots to the level in the wild-type. These results further support the idea that a certain level of intracellular auxin plays a critical role in regulating the ethylene-mediated growth response (Rahman et al. 2001b). It is noteworthy that application of NAA on its own at a concentration of 10 nM did not inhibit root growth, but this was the optimum concentration for recovery of the ethylene response in the mutant roots. This indicates that a low level of auxin is enough for the normal ethylene response in contrast to auxin-induced inhibition of growth.

Since the auxin uptake is reduced in *aux1-7* mutant, it is logical to assume that the intracellular level of auxin is lower in the roots of this mutant compared to the wild-type. The defect in auxin efflux in *eir1-1* roots reduces the basipetal transport of auxin from root tip to elongation zone (Rashotte et al. 2000), which could result in a reduction of intracellular level of auxin in the elongation zone. Initially, it was proposed that CSI inhibits ethylene signaling (Rahman et al. 2000); however, this hypothesis can be modified to the idea that auxin is a positive regulator for ethylene-mediated inhibition in root elongation (Rahman et al. 2001b) and that CSI regulates ethylene response in roots by modulating the auxin concentration in root cells (Fig. 4; Rahman et al. 2001a).

CSI stimulates the growth of horizontal roots but not vertical ones : CSI stimulated the elongation of *Arabidopsis* roots grown horizontally on wet filter paper by several-folds (Rahman et al. 2000). However, when *Arabidopsis*

seedlings were grown on vertically oriented agar plates, CSI did not show any significant stimulatory effects on the growth of wild-type roots, although CSI disrupted the direction of root growth (Rahman et al. 2001a; Fig. 2). This variation in CSI action is due to the difference in involvement of ethylene in regulating root elongation. On filter papers, the length of wild-type roots was much shorter compared to ethylene insensitive mutant *ein2-1* roots (Rahman et al. 2000), whereas the length of roots of both genotypes is not significantly different from each other, when grown vertically (Rahman et al. 2001a). These

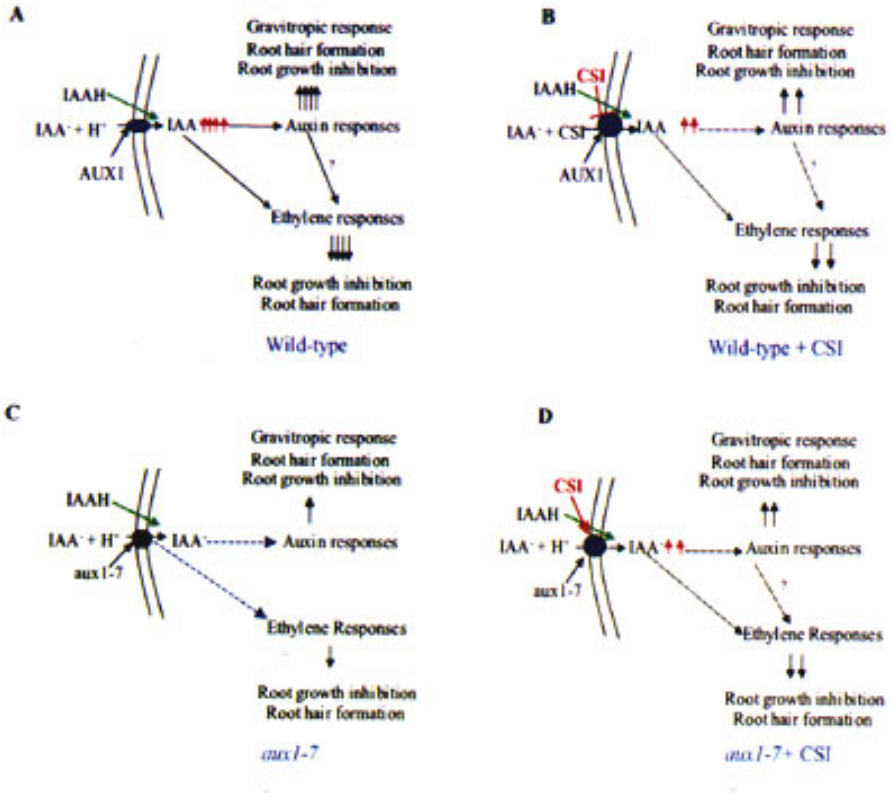

Fig. 4. Schematic representation of the role of AUX1 in regulating the intracellular level of auxin and both auxin and ethylene responses in the roots of wild-type (**A**, **B**) and *aux1-7* (**C, D**). CSI specifically interacts with the wild-type AUX1 protein (**B**) or with mutated aux 1-7 protein (D). CSI induces a change in the conformation of the protein resulting in a modulation of auxin uptake in root cells, which in turn regulates both auxin and ethylene responses. Diffusion of auxin (represented by green broken arrows) into the root cells is constant in all cases. Solid and broken arrows indicate strong and weak signals, respectively, and blue arrows are weakest. The number of black and red arrows represents the extent of hormone response and the level of auxin concentration, respectively.

results indicate that the endogenous ethylene plays a major role in inhibition of the elongation in wild-type roots grown horizontally, whereas it does not show this tendency in the vertical condition. Hence, CSI does not stimulate the elongation of vertical roots. The bioassay system using filter paper is very simple and convenient but consideration of the involvement of ethylene in this type of root-growth assay is essential.

CSI mimics a loss of function in AUX1 gene : The auxin influx mutant *aux1-7* shows a reduced IAA uptake, an agravitropic nature in roots and strong resistance to ethylene in root elongation. The phenotypes of this mutant are very similar to those of CSI-treated wild-type seedlings (Rahman et al. 2001a), indicating that CSI mimics a loss of function in *AUX1* gene. The interaction of CSI with AUX1 protein was confirmed by the fact that CSI inhibited IAA uptake but not NAA in wild-type roots (Fig. 3; Rahman et al. 2001a). Furthermore, our recent experiments have shown that CSI inhibits IAA-induced expression of β-glucuronidase (GUS) gene but not the one induced by NAA in an auxin-dependent GUS reporter line (Rahman et al. 2002).

CSI is an effector of G protein-mediated sugar signal transduction cascade in invertebrates : CSI is a sweet substance for the blowfly, *Phormia regina* (Ahamed et al. 2000, 2002), although not sweet for human. Application of CSI-induced feeding in the blowfly results in impulse generation in the sugar taste receptor cells at concentrations hundred times less compared to sucrose. In the behavioral and electrophysiological studies of the sugar taste receptor cells of blowfly, both the feeding response and the impulses appeared after a relatively long latency, suggesting that CSI actually penetrates into the sugar taste receptor cells and thereby activates the sugar signal transduction cascade. The effects of CSI were completely inhibited by a nonhydrolyzable GDP analogue GDPβS, an inhibitor of G protein, indicating that CSI-induced sugar signal transduction involves a G protein-mediated cascade (Ahamed et al. 2002). Although the CSI molecule contains a sugar chain, the sugar taste of CSI is unlikely to be due to the sugar groups (Ahamed et al. 2000). Recent findings suggest that G protein plays an important role in the growth and development of *Arabidopsis* (Ullah et al. 2001; Chen 2001). At present, it is not clear whether or not the CSI action on G protein-mediated signal transduction cascade has any relation to the role of CSI as an auxin influx modulator; only future research may shed light on the link between these two apparently different actions of CSI.

Interaction of CSI with AUX1 protein : The effect of CSI on *aux1-7* roots is novel: CSI increases the uptake of auxin and restores both gravitropic and ethylene responses. The CSI-induced restoration of gravitropic response in *aux1-7* roots is allele-specific, not observed in other *aux1* alleles (data to be published elsewhere). The *aux1-7* mutant is a missense mutant where Gly^{459} is changed to Asp^{459}close to the carboxyl terminal end (Bennett et al. 1996). It has been shown by using anti-sera on Western blots that the full-length protein is expressed in mutant roots to the same level as that of the wild-type, yet it acts as a complete loss-of-function mutant (personal communication from Dr. Bennett). How CSI inhibits the auxin influx carrier AUX1 and stimulates the mutated aux1-7 carrier protein is an enigma. There are several possibilities but the most attractive one is being explained schematically in the following figures (Fig. 4).

By facilitating the uptake of auxin, AUX1 helps maintain a supra optimal level of auxin in the wild-type root cells. This intracellular level of auxin also plays an important role in regulating ethylene-mediated responses in the growth of roots and root hair formation (Masucci and Schiefelbein 1996; Rahman et al. 2002). Auxin uptake by the influx carrier AUX1 and the regulation of both auxin and ethylene responses in the wild-type roots are shown schematically in Fig. 4A. In *aux1-7* roots, the intracellular level of auxin drops to a sub-optimal level because of a reduction in auxin influx, which results in a reduced response to both auxin and ethylene (Fig. 4C).

CSI negatively regulates the AUX1 protein in the wild-type roots (Fig. 4B). One possible mechanism may be as follows : CSI interaction with this protein may change the conformation of the protein from an active- to a less active state. A auxin uptake is therefore considerably less compared to the untreated wild-type (Fig. 3A). The CSI-induced inhibition in auxin influx capacity of AUX1 results in a decrease in intracellular level of auxin, which in turn reduces both auxin and ethylene responses of the wild-type roots (Fig. 4B).

In *aux1-7* roots, CSI interacts with the mutated aux1-7 protein in the same manner as the wild-type AUX1 protein and changes the conformation of the mutated protein from an inactive state to a more active-form. (Fig. 4D). The CSI-induced change in the protein conformation results in a restoration of auxin influx to some extent, which in turn increases the intracellular level of auxin in this mutant root. The increase in auxin concentration partially restores the

gravity response and the ethylene response of *aux1-7* roots. It is noteworthy to mention that both the gravitropic response and the level of auxin influx in CSI-treated *aux1-7* roots are almost similar to those in CSI-treated wild-type roots (Rahman et al. 2001a). This observation also supports the hypothesis that the CSI interaction with either the wild-type AUX1 or mutated AUX1 results in a similar conformational change of the protein (Figs. 4B and D).

Conclusion : Auxin efflux carriers play an important role for auxin transport. A group of flavonoids including quercetin have been shown to be natural inhibitors for auxin efflux (Brown et al. 2001). To our knowledge, CSI is the first natural compound to modulate the auxin influx carrier AUX1. In addition to gravitropic response in auxin transport, recently AUX1 has been shown to play several roles in plants including development of lateral root primordium (Casimiro et al. 2001), IAA loading into the leaf vascular transport system and IAA unloading in the primary root apex (Marchant et al. 2002). Since the endogenous auxin level in plant cells is an important factor in regulating growth and development of plants, CSI could prove to be a powerful tool in orchestrating plant development by regulating auxin concentration and ethylene signaling in the root.

Acknowledgements

The authors thank Professor A. S. Islam, the Chief Editor of Plant Tissue Culture, for invition to write this review, and Dr. Malcolm J. Bennett of Nottingham University for providing them the *aux1* seeds used in the study and also for invaluable suggestions.

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